# A VARIANT OF RIFT VALLEY FEVER VIRUS

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Two strains of an agent which appears to be either atypically reacting Rift Valley fever (RVF) virus, or a close relative to it, have been isolated from mosquitoes caught in the Lunyo Forest on the Entebbe peninsula. The first strain to be isolated was obtained from a mixed pool of mosquitoes of the genus *Aëdes* and subsequent work has produced evidence that the agent was derived from *Aëdes* (*Banksinella*) *circumluteolus* Theobald. The other strain was obtained about 10 months later from *Aëdes* (*Stegomyia*) *africanus* Theobald caught under similar circumstances in the same area.

Part I of this communication records the isolation of these two strains while Part II describes the results of experiments which were designed to identify them and to show the differences between the locally isolated agent, referred to in this paper as 'Lunyo virus', and classical Rift Valley fever virus.

## PART I. ISOLATION

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Detailed descriptions of the routine processes used in this laboratory for attempted virus isolation have recently been given in papers on the isolation of Chikungunya and Zika viruses.[1,2] In view of this, it will suffice here to describe the strains of known RVF virus used in certain experiments:

*Pantropic RVF Virus*

This is a strain of virus isolated from mosquitoes, *Eretmapodites* spp., caught in Bwamba County, Uganda.[3] It had undergone approximately 160 mouse passages and was used in the form of infective mouse serum.

*Neurotropic RVF Virus*

This was a strain of RVF virus which had undergone approximately 100 brain to brain passages in mice.

### MOSQUITO-CATCHING PROGRAMMES

All the mosquitoes were adult females taken when they alighted on human bait and were caught by a team of trained African youths working under supervision. Each catching period lasted 2½ hours and usually took place during the forenoon, but sometimes in the afternoon and on occasions twice in the same day. The mosquitoes were brought to the laboratory at the end of each catch, in their individual catching tubes. They were then identified and those intended for inoculation were chilled at 4°C to immobilize them and pooled by species. The pooled mosquitoes were then stored at −20°C until suspensions could be made for inoculation into experimental animals.

The first catching programme took place between 26 October and 18 November 1955, during which period 4,130 mosquitoes were inoculated in 29 pools. These mosquitoes are listed below according to the nomenclature used by Edwards.[4]

| | |
|---|---|
| *Anopheles* (*Anopheles*) *implexus* Theobald | 12 |
| *Hodgesia cyptopus* Theobald | 10 |
| *Taeniorhynchus* (*Coquillettidia*) *metallicus* Theobald | 129 |
| *pseudoconopas* Theobald | 697 |
| *maculipennis* Theobald | 117 |
| *fuscopennatus* Theobald | 212 |
| *aurites* Theobald | 952 |
| (*Mansonioides*) *africanus* Theobald | 55 |
| *uniformis* Theobald | 5 |
| *Aëdes* (*Mucidus*) *nigerrimus* Theobald | 1 |
| (*Finlaya*) *ingrami* Edwards | 4 |
| (*Stegomyia*) *apicoargenteus* Theobald | 13 |
| *africanus* Theobald | 100 |
| (*Aëdimorphus*) *argenteopunctatus* Theobald | 4 |
| *domesticus* Theobald | 1 |
| *leptolabis* Edwards | 20 |
| *tarsalis* Newstead group | 4 |
| *cumminsi* Theobald | 11 |
| sp. indet. | 1 |
| (*Banksinella*) *circumluteolus* Theobald | 1,015+493=1508 |
| *Eretmapodites chrysogaster* Graham group | 21 |
| *oedipodius* Graham group | 125 |
| *Culex* (*Lutzia*) *tigripes* Grandpré and Charmoy | 13 |
| (*Culiciomyia*) *nebulosus* Theobald | 1 |
| *cinereus* Theobald | 2 |
| (*Culex*) *annulioris* Theobald | 25 |
| *univittatus* Theobald | 5 |
| *Culex* (*Culex*) *quasiguiarti* Theobald | 63 |
| *decens* Theobald | 5 |
| *perfidiosus* Edwards group | 2 |
| *guiarti* Blanchard | 9 |
| *moucheti* Evans | 3 |
| | 4,130 |

In the above list the figures in the right-hand column represent, for all species except *A. circumluteolus*, the total number of mosquitoes caught in 1 afternoon and 19 morning catching periods. During this period the catching team numbered 6, and the figures represent the results of 300 man-hours of catching. The multiple figure for *A. circumluteolus* represents the total as for the catches mentioned above, together with the number of these insects caught during 4 extra morning and 4 extra afternoon sessions. These extra sessions were undertaken when it became apparent that an isolation of virus had been obtained from this species. Towards the middle of November the number of insects taken at each catch fell markedly, probably owing to a seasonal variation, and the programme was brought to an end.

When it was not feasible to inoculate the species processed individually, suspensions were pooled but, though species might be combined in one inoculum, the genera were always kept separate. In those cases in which there was a relatively

large number of mosquitoes of one species to be mixed with a small number of mosquitoes of other species, individual suspensions were made, after which aliquots of each were taken and mixed to form the inoculum. The remainder of each suspension was stored separately, frozen at −20°C to form reference material.

The second catching programme was organized between 9 May and 27 August 1956 in order to attempt virus isolations from *A. africanus*; all other biting insects taken were discarded. During the course of this programme 1,355 *A. africanus* were collected in 80 catching periods and were inoculated as 11 pools. To differentiate these pools from the previously mentioned ones (which were numbered with Arabic numerals), they were designated by Roman numerals. Three strains of virus were isolated during this programme. Pool Lunyo III yielded a strain of Lunyo virus and Pools Lunyo V and VI yielded strains of Zika virus.[2]

### THE FIRST ISOLATION OF LUNYO VIRUS

This was obtained from Pool 8, which consisted of equal parts of 2 suspensions made on 5 November 1955 from mosquitoes caught between 31 October and 4 November. The composition of the 2 suspensions is shown below:

| | | | |
|---|---|---|---|
| Suspension A: | 468 | *A. circumluteolus* | in 10 ml. diluent |
| Suspension B: | 1 | *A. nigerrimus* | in 2·0 ml. diluent |
| | 2 | *A. ingrami* | |
| | 1 | *A. apicoargenteus* | |
| | 3 | *A. argenteopunctatus* | |
| | 4 | *A. leptolabis* | |
| | 1 | *A. tarsilis* group | |
| | 2 | *A. cumminsi* | |

The Pool-8 mixture was inoculated into 2 groups of 5 3-day-old mice and 1 group of 5 adult mice. On the 4th post-inoculation day 1 infant mouse was missing from each litter; on the 6th day, in 1 litter 1 infant was missing and another dead, and in the other litter 2 infants were partly eaten. The brains of the dead mouse and of 1 partially eaten infant mouse were salvaged and used to make passages which were successful both before and after the suspension had been passed through a Seitz EK filter pad. The remaining 4 infant mice were sacrificed when sick and their brains were stored in glycerol saline at −20°C. On the 7th day of checking 1 adult mouse was dead; its brain was removed and stored in glycerol saline until 16 February 1956, when it was used to initiate a successful passage series of an agent later shown to be immunologically identical with that obtained from the infant mice. The rest of the adult mice remained well and apparently unaffected by the inoculum.

When the infant mice mentioned above were found to be succumbing to the effects of the inoculum, the stored aliquots of the two mosquito suspensions which were used to make Pool 8 were thawed and inoculated separately into mice. The *A. circumluteolus* suspension was inoculated into 8 litters of 3-day-old mice and 2 groups of adult mice. The other suspension was used to inoculate 2 groups of infant mice and 1 group of adults. None of the mice showed any sign of illness. They were not, however, discarded after the usual observation period, and 6 weeks after they had received their inoculation they were divided into groups, some of which received challenging doses of 3·2 or 32 $LD_{50}$ of 5th-passage Lunyo virus, while the other groups received 63 or 630 $LD_{50}$ of 18th-passage virus. Control groups, consisting of mice of the same age received similar challenges. All the mice which received the larger challenge of 5th-passage virus or any 18th-passage virus died. Of the mice which received the challenge of 3·2 $LD_{50}$ of virus, all those in the control groups died whereas no mice died in 2 groups which had been inoculated as 3-day-old infants with the original *A. circumluteolus* suspension. One of the groups of 5 adult mice which received inocula of this suspension was also challenged with 3·2 $LD_{50}$ of virus, as a result of which 2 died but 3 lived. On 22 November 1955 the remaining 2·8 ml. of the *A. circumluteolus* suspension was thawed and inoculated subcutaneously into rhesus monkey MR 1126. This monkey showed no signs of illness but 3 weeks later its serum neutralized $10^{0·8}$ $LD_{50}$ of Lunyo virus as compared with the pre-inoculation sample, and at 6 weeks after the inoculation it was able to neutralize $10^{1·5}$ $LD_{50}$ of the virus.

The evidence given above concerning the production of a neutralizing substance for Lunyo virus in mice and in monkey MR 1126, after the inoculation of the suspension of *A. circumluteolus*, lends support to our belief that Pool 8 was the origin of the agent although it failed to yield a re-isolation of virus. The fact that, after the original inoculation, the infant mice did not become ill until the 6th day of checking and that only 1 of the adult mice became ill may indicate that there was only a small quantity of active virus in the original suspension, especially as both infant and adult mice, inoculated with material derived from the first mouse groups to be inoculated, were all dead on the 3rd post-inoculation day, thus showing that the agent readily adapts to mice. A further point which was against obtaining a re-isolation is the marked instability of the agent when stored as a frozen suspension of material in bovine plasma albumin (Armour's fraction V, a 0·75% solution in a phosphate-buffered saline pH 7·4. Hereafter in the text this is referred to as BPA). This will be described in more detail in Part II of the communication.

### THE SECOND ISOLATION OF LUNYO VIRUS

This was obtained from a suspension of 28 *A. africanus* prepared on 5 June 1956.

This suspension was inoculated into 2 litters of 3-day-old mice, 1 group of adult mice and rhesus monkey No. 1078. On the 7th post-inoculation day 1 of the infant mice died, but the carcase was eaten by the mother and material for passage could not be obtained. None of the other inoculated mice succumbed during a 30-day observation period. Also on the 7th post-inoculation day MR 1078 was found to have a fever of 104°F and was bled. By the following day the monkey's fever had subsided, but it rose again on the 10th post-inoculation day to 104°F, when it was bled again. Isolations of virus were made from both these blood specimens when serum from them was inoculated into mice. On the 8th post-inoculation day it became apparent that no more infant mice of the original groups were likely to be affected by the inoculation, and the stored suspension was thawed and used to inoculate 4 litters of newborn mice in the hope that they would prove more susceptible than 3-day-old mice and would provide an isolation of virus. In 3 of the 4 groups of newborn mice no sign of illness was detected during a 30-day observation period. In the remaining group, however, the mother ate 1 infant on each

of the 5th and 6th post-inoculation days. On the 7th post-inoculation day the mother was found dead and a foster mother was provided for the 3 weak infants which remained. On the 8th post-inoculation day 1 infant had been eaten and on the 9th post-inoculation day the remaining 2 infants were found to be ill. The brains were removed from these ill infant mice and were subsequently found to contain an agent which was shown to be very closely related to the agent isolated from *A. circumluteolus*, if not identical with it, as were the isolates obtained from the serum of monkey 1078.

From this it appears that there was a strain of virus in the *A. africanus* pool which, though it readily infected the rhesus monkey, was only able to induce an apparent infection in very young suckling mice which were rendered more than usually susceptible by reason of intercurrent illness of the mother which led to her death and the consequent starvation and chilling of the infants.

## PART II. IDENTIFICATION AND PROPERTIES

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### *The clinical picture produced in mice*

When inoculated into infant mice Lunyo virus kills them rapidly without the production of unusual symptoms or signs. In adult mice, however, the agent produces a characteristic clinical picture. The mice become markedly hyperactive and when disturbed rush about the cage, occasionally making great leaps so that, when inspecting them in the earlier stages of an infection, the examiner has to exercise great care not to allow a mouse to leap out of the box. This phase is followed by a quieter one, in which the mice sit up with the tail stretched out behind them, rather in the attitude of a kangaroo, while industriously eating any available material. This material is apparently taken without any form of selection, its availability being the most important consideration, and the animals may frequently be seen consuming the bedding or even the carcase of another member of the group which has succumbed more rapidly, though an adequate supply of normal mouse food is within easy reach. Some of the animals appear to suffer from localized areas of irritation or pain; they claw at their own lower jaw and throat, sometimes inflicting severe wounds in the process, while others attack a thigh, or, possibly more often, a fore-limb, when it is not unusual for them to nibble off the entire paw. Mice in more advanced stages of the illness are often seen in convulsions and, in fact, it would appear that the majority of those which die do so while in convulsions. In cases where the animals receive small doses of virus and the survival time is prolonged, paralysis may occur.

### *Susceptibility of mice to parenteral inoculation of Lunyo virus as isolated*

It was noted that Lunyo virus did not affect 5-day-old mice to the degree expected when they were inoculated IP* with 1st-passage material. The results of the experiment described below confirm that, as isolated, Lunyo virus shows a considerable difference in potency when aliquots of the same dilutions are inoculated by the IC† and IP routes.

* IP = intraperitoneal(ly). † IC = intracerebral(ly).

A $10^{-1}$ Seitz-filtered 4th-passage infant-mouse brain suspension was used to make decimal dilutions down to $10^{-7}$: these were used to inoculate groups of 5 adult mice by the IC and IP routes. The results are shown below:

| | INTRACEREBRAL ROUTE | | | INTRAPERITONEAL ROUTE | | |
|---|---|---|---|---|---|---|
| *Dil.* | *Dead* | *Alive* | *Titre* | *Dead* | *Alive* | *Titre* |
| $10^{-1}$ .. | 5 | 0 | | 2 | 2 | |
| $10^{-2}$ .. | 5 | 0 | | 3 | 2 | |
| $10^{-3}$ .. | 5 | 0 | $\geq 7{\cdot}3$ | 4 | 1 | 2·7 |
| $10^{-4}$ .. | 5 | 0 | | 0 | 5 | |
| $10^{-5}$ .. | 5 | 0 | | 0 | 5 | |
| $10^{-6}$ .. | 4 | 0 | | 0 | 5 | |
| $10^{-7}$ .. | 3 | 1 | | 0 | 5 | |

From the above results it will be seen that there is not only a marked difference in the titre obtained, but also that the mice which do die after IP inocula do not appear to be more likely to do so if given a dose more than 100 times as great as the minimum infecting one.

### *The occurrence of Lunyo virus in the blood and liver of mice during an intracerebral passage series*

In a number of cases mice infected with Lunyo virus show signs of liver damage. It was decided to investigate the relative amounts of virus present in the brain, blood and liver both early and late in the course of an IC passage series, which was likely to have enhanced the neurotropic quality of the agent at the expense of any viscerotropic tendency in the original isolate. The results of parallel titrations of the 3 types of material from 3 different sources of moribund mice are shown below and, from them, it may be seen that at the 24th passage, though no virus is recoverable from the blood, there is still some activity in the liver:

| *Passage levels* .. | 4 | 5 | 24 |
|---|---|---|---|
| Brain .. .. | 6·0 | 6·6 | 6·5 |
| Liver .. .. | 3·6 | 4·6 | 2·6 |
| Blood .. .. | 1·2 | 1·8 | 0 |

### *Enhancing the viscerotropic element of Lunyo virus*

As isolated, Lunyo virus would appear to be neurotropic rather than viscerotropic though in many, if not all, infected mice there was evidence of some activity in the liver. It was felt that in order to increase the tendency for the agent to grow in the liver, the intraperitoneal route of inoculation should be used, and only livers which showed macroscopic change should be selected to provide material for passage.

A passage series was initiated using 2nd-passage infant-mouse liver which had been stored at −20°C in glycerol saline. This was suspended in BPA and used to inoculate infant mice IP. When they sickened, a number were sacrificed and from them the 3 most markedly affected livers were chosen to make the next passage, which was to both infant and adult mice. When these succumbed all were found to have very pale mottled livers and the clinical picture in the adult mice was less obviously encephalitic. Again the livers showing the greatest degree of change were taken for passage. When the next passage was due it was noticed that the mice showed no encephalitic signs but were dying in a state of prostration within 3 or 4 hours of the first signs of illness. When the passage was made, it was done in the form of two

titrations, one of pooled liver and the other of pooled blood. The liver gave a titre of $10^{5\cdot8}$ and the blood $10^{8\cdot2}$ $LD_{50}/0{\cdot}03$ ml. After this only one more transfer of liver was made. This was from moribund mice in the liver titration; it was in turn set up as a titration, which gave a figure of $10^{7\cdot8}$ $LD_{50}/0{\cdot}03$ ml. From the titration of blood another continuous IP passage series was started in which mouse serum was used as passage material, and after only a few passages the behaviour of the agent was indistinguishable from classical RVF though, as will be shown later, it could be induced to revert to the original pattern very rapidly.

*Attempts to neutralize early passage Lunyo virus with known immune sera*

On the third post-inoculation day, in the adult group inoculated with Seitz-filtered material derived from the first mouse to succumb to the Pool 8 suspension, 4 mice were dead and 1 was moribund. The dead mice appeared relatively fresh, and their brains were removed and saved for reference material; the moribund mouse was taken for passage. Following the removal of the brains of these mice, the carcases were opened for a routine post-mortem check. One of the dead mice had a pale liver and the moribund mouse had a very pale mottled liver; a specimen of heart blood taken into a capillary tube failed to clot at 20 minutes. In view of these findings, when the routine IC passage of brain material was made to 2 litters of infant and 1 group of adult mice, a spot test was made with known potent RVF antiserum. In the test $10^{-1}$ and $10^{-4}$ dilutions of the brain suspension were mixed with equal parts of RVF antiserum and, after incubation of the mixtures (together with controls in which BPA was used in place of immune serum) at 37°C for 1 hour, they were inoculated into litters of 5-day-old mice by the IP route. In the control groups all the mice died at $10^{-1}$ and 1 of 5 died at $10^{-4}$; in the test groups all the mice died at $10^{-1}$ and all survived at $10^{-4}$. The potency of the material was lower than might be expected of a virus which kills adult mice within 72 hours but, as will be shown later, the early-passage material was in a form that does not readily infect mice by the IP route. It was nevertheless felt that, even though the $10^{-4}$ dilutions gave no definite information, the fact that there was no difference between the $10^{-1}$ groups was significant, since the RVF immune serum was known to neutralize at least $10^{8}$ $LD_{50}$ of the homologous virus when tested in adult mice by the IP route.

When it appeared that the test mentioned above was unlikely to prove positive, attention was directed towards Bunyamwera virus, for this was the only agent which was known to give a clinical picture even remotely resembling that of Lunyo virus in adult mice though, in Bunyamwera infection, when a clawing action was seen it was invariably directed towards the nose.

A Seitz-filtered suspension of 4th-passage material was used to set up titrations in BPA and Bunyamwera immune serum. The results showed that there is no inhibition of the action of Lunyo virus by antibodies to Bunyamwera virus.

A more comprehensive test for possible interaction of Lunyo virus and RVF antibodies was then carried out. A suspension of 8th-passage material was prepared and parallel titrations set up, BPA being used as control and a potent human RVF antiserum as test. This antiserum was chosen because it had the highest neutralization index of all the RVF antisera available. In an IP test an aliquot of the same sample had been shown to neutralize more than $10^{8\cdot7}$ $LD_{50}$ of virus. For this test 2 hours' incubation at 37°C was used and the material was inoculated IC into adult mice. The figures obtained for BPA and the test serum were $10^{5\cdot7}$ and $10^{5\cdot3}$ respectively and were taken to indicate that no interaction between virus and antibody was likely to have occurred.

Other known potent immune sera which were tested and found to be devoid of neutralizing power for Lunyo virus were those prepared against Uganda S, Semliki Forest, Pongola,* Spondweni,* Wesselsbron,* West Nile, yellow fever, Bwamba fever, Zika, Chikungunya, Nairobi sheep disease, and Mengo viruses.

A specimen of Lunyo virus was sent to Dr. Max Theiler, of the Rockefeller Foundation laboratories in New York. He reported that though no haemagglutinin had been produced for it, a complement-fixing antigen had been readily obtained. This failed to react with any of the African group-A or group-B antisera, or with antisera to Oriboca, Marituba, sandfly, Bwamba, Bunyamwera, Simbu, blue tongue or horse-sickness viruses. It did, however, react strongly with the homologous antiserum and an antiserum prepared against RVF in Johannesburg by Dr. R. H. Kokernot. In view of these findings, Dr. Theiler felt that there was no doubt that the Lunyo virus is identical or closely related to RVF and, because of this, he decided that no further work with Lunyo virus could be carried out in his laboratory.

*Cross neutralization of the viscerotropic strain of Lunyo and RVF*

During the period between isolation and the production of the viscerotropic strain of Lunyo, a test had been set up in which specific Lunyo antisera was used against a RVF challenge. This test showed that, even though the Lunyo virus was not apparently affected by RVF antiserum, the reverse was not the case, the Lunyo antisera, whether derived from monkey or rabbit, proving able to neutralize RVF virus in approximately the same degree as homologous antiserum. In view of this finding, and of Dr. Theiler's report from New York, when the viscerotropic strain of Lunyo became available a test was planned in which this agent and classical RVF were used in titrations

TABLE I. RESULTS OF CROSS NEUTRALIZATION TESTS WITH VISCEROTROPIC LUNYO AND RVF VIRUSES (EXPRESSED AS LOGS. VIRUS NEUTRALIZED)

| Challenge / Antiserum | RVF IP | Lunyo IP | RVF IC | Lunyo IC |
|---|---|---|---|---|
| Titre of virus .. .. | $10^{8\cdot5}$ | $10^{7\cdot5}$ | $10^{8\cdot7}$ | $10^{8\cdot4}$ |
| Lunyo monkey .. | 6·0 | 7·0 | 3·0 | 1·9 |
| Lunyo rabbit .. .. | 7·8 | 7·0 | | |
| RVF human .. .. | 7·5 | 7·0 | 3·0 | 2·2 |
| RVF rabbit .. .. | 7·8 | 6·8 | 3·0 | 3·0 |

* These are agents recently isolated in South Africa and antisera to them were provided by Dr. K. C. Smithburn of the Rockefeller Foundation.

of antisera to both, inoculated by both the IC and IP routes. The results of this test are set out in Table I, from which it may be seen that there is a quantitative and reciprocal cross reaction. One may, therefore, assume that in this case the challenging viruses are strains of the same agent.

*The degree to which neurotropic and viscerotropic strains of virus may be neutralized*

It may be seen in Table I that when inoculated by the IC route a lesser degree of neutralization is obtained for both strains of virus as compared with the IP route. It was at first thought that the relatively low neutralization indices obtained in antisera produced for the original Lunyo isolate, whether in monkeys or rabbits, might have been due to the fact that the agent is a poor antigen. That this is not the case was shown when these antisera were found to be very potent for RVF. Returning to the data in Table I, it may be seen that, although when the IP route is used the viscerotropic strain of Lunyo virus is neutralized by all the antisera to a degree comparable to that obtained in the titrations against RVF, when the IC route of inoculation is used the antisera are able to neutralize less of the Lunyo virus than of RVF. It is thought that this might be because a proportion of the inoculum immediately resumes the neurotropic character. This theory is supported to some extent by the fact that the mouse groups in the Lunyo IC titrations gave a somewhat longer average survival time than in the RVF IC titrations, and many of them, especially at the lower dilutions, showed the encephalitic syndrome described for the original isolate.

In an attempt to discover whether the phenomenon of 'poor neutralizability' of a neurotropic strain was true for RVF as well as Lunyo, titrations of neuro-adapted RVF virus were set up in various antisera using the IC route in adult mice. The results of these titrations are shown below:

| | |
|---|---|
| BPA control .. .. .. .. .. .. | $10^{-6\cdot6}$ |
| RVF immune rabit serum .. .. .. .. | $10^{-6\cdot4}$ |
| Lunyo immune rabbit serum .. .. .. .. | $10^{-6\cdot6}$ |

The fact that neither of the rabbit antisera was able to neutralize the neurotropic RVF virus when inoculated by the IC route in adults casts doubt on the identity of the virus used. However, when tested by the IP route in infant mice the following results were obtained:

| | |
|---|---|
| BPA control .. .. .. .. .. .. | $10^{6\cdot9}$ |
| RVF immune rabbit serum .. .. .. .. | $10^{2\cdot0}$ |

The results of this experiment show that, as with Lunyo virus, the neuro-adapted form of RVF is much less readily neutralized by an antiserum than the viscerotropic form. Again as with Lunyo virus, an antiserum prepared in rabbits to the neuro-adapted RVF was able to neutralize less of this strain than of the viscerotropic RVF.

*Transmission of Lunyo virus by the bite of a mosquito*

Early in 1956 a programme designed to show whether Lunyo virus could be transmitted by the bite of various *Aëdes* spp. was begun in collaboration with Dr. A. Michael Davies.* Shortly after the inception of the work, however, Dr. Davies was taken ill and returned to Israel, after which the programme lapsed, owing to which only 1 species of mosquito, *Aëdes* (*Stegomyia*) *aegypti* (Linnaeus), was used and, though the planned experiments were not all completed, it is of interest to quote certain of the results obtained, because they show that this mosquito is capable of transmitting the virus, whereas attempts to induce it to transmit RVF have not been successful.[5,6]

Adult female *A. aegypti* drawn from the Institute insectary were allowed to bite a mouse which was moribund after an intraperitoneal inoculation of Lunyo virus that had undergone 1 IC brain passage and 5 consecutive IP passages of liver. The feeding of the mosquitoes was carried out by placing the mouse, which had been immobilized by the intraperitoneal inoculation of 0·15 ml. of a 2·5% solution of bromethol in saline, into the Barraud cage in which the mosquitoes were kept. After an elapsed time of 1 hour the mouse was removed and sacrificed, and titrations were made of blood, liver and brain; these respectively gave titres of $10^{6\cdot7}$, $10^{5\cdot4}$ and $10^{3\cdot8}$ mouse $LD_{50}$/0·03 ml. inoculum.

After the feed, all the mosquitoes which contained blood were transferred from the Barraud cage to individual 3 inch × 1 inch tubes.[7] After 20 days, 25 of the fed mosquitoes, which had, in the meantime, been maintained on a raisin diet, were each given the opportunity of feeding on 5-day-old infant mice. Three infant mice were used, each being identified by marking, and the following record was kept of the number of mosquitoes which probed and the number which actually fed on each mouse:

| | *Mouse No.* 1 | *Mouse No.* 2 | *Mouse No.* 3 |
|---|---|---|---|
| No. mosquitoes probed | 5 | 6 | 4 |
| No. mosquitoes fed .. | 3 | 2 | 5 |

After the feeding, the 3 infant mice were returned to the box along with the mother and their 2 litter mates, which acted as controls to cover the possibility of death from intercurrent illness.

The 25 mosquitoes were triturated in 2·0 ml. of BPA and the resulting suspension titrated in mice, when a figure of $10^{5\cdot4}$ $LD_{50}$/0·03 ml. was obtained.

The infant mice were observed 6-hourly for 5 days and then 12-hourly for a further 10 days. During this period the 2 normal controls showed no sign of illness but at 66 hours mouse No. 1 was sick and Nos. 2 and 3 were dead and had been partly eaten, although the brains were saved. Mouse No. 1 gradually recovered and was well when discarded on day 15. Two weeks later, the brains which had been removed from mice Nos. 2 and 3 and stored in glycerol saline were used to set up an IC identification test in adult mice. The results of this test are given below:

| *Antiserum* | *Logs Virus Neutralized* |
|---|---|
| RVF (human) .. .. .. | 1·7 |
| RVF (rabbit) .. .. .. | 1·9 |
| Lunyo (monkey) .. .. .. | 3·0 |
| Lunyo (rabbit) .. .. .. | 2·4 |

From these results it may be seen that the mice died of a strain of Lunyo virus presumably acquired through the bite of *A. aegypti*. An interesting fact is that, though the mosquitoes were infected by feeding on mice circulating the viscerotropic type of virus, the mice inoculated with suspensions of these mosquitoes, or with material from the infant mice which died following mosquito-bite, all showed the characteristic signs of an infection by the neurotropic type of Lunyo virus. This is borne out by the figures given

* A visiting worker from the Hebrew University, Jerusalem.

for the identification test above, for one would expect the viscerotropic strain to be neutralized at least equally well by antisera to RVF and possibly better by the RVF rabbit (see Table I), whereas the Lunyo antisera were more potent than the RVF ones.

It is worthy of note that an almost identical attempt to effect transmission on the 8th day after the mosquitoes fed on the infective mouse failed, though the suspension subsequently made of the mosquitoes gave a titre of $10^{6\cdot6}$ $LD_{50}$/0·03 ml. being 1·2 logs higher than in the successful transmission.

*Stability of Lunyo virus*

The agent loses, on an average, a little more than 1·0 log of potency in the process of lyophilization. If the preparation for drying is made in BPA, then the dried virus undergoes considerable further loss on storage at −20°C. When made up as a suspension in non-immune serum, a dried preparation stores much better. When stored frozen at −20°C, a suspension of brain in BPA loses all potency in a short time. A suspension made in BPA was titrated and found to contain $10^7$ $LD_{50}$/0·03 ml. After lyophilization and storage at −20°C for 18 days, the titre fell to $10^{2\cdot8}$ $LD_{50}$/0·03 ml. and another portion, which was not lyophilized but was stored frozen for the same period, contained no active virus. A preparation of brain in 100% normal serum titred $10^{6\cdot8}$ on manufacture, and the titre fell to $10^{4\cdot5}$ after lyophilization and storage at − 20°C for 6 weeks.

The viscerotropic adaptation of Lunyo virus made possible the collection of infective mouse serum for storage as stock material. This was considerably more stable than the brain suspensions mentioned above. Serum which gave a titre of $10^{-7\cdot0}$ when stored at −20°C dropped to $10^{-5\cdot8}$ over a period of 28 days. Thus even when stored under the optimum condition of suspension in 100% serum, the Lunyo virus is less stable than classical RVF, for which Mims[8] reports no loss in potency after storage for 33 days at −20°C.

It was discovered that the original Lunyo isolate was most likely to remain viable after a long period if stored as small pieces of infant mouse brain in 50% glycerol saline.

*Lunyo virus in monkeys*

Rhesus monkey *Macacus mulatta* Zimmerman No. 1131 was bled and its serum tested for antibodies to Lunyo virus. When the results of this test confirmed that the monkey was non-immune it was inoculated subcutaneously with 1·0 ml. of $10^{-1}$ Seitz-filtered 5th-passage mouse-brain suspension, a titration of which was set up within a few minutes of the monkey inoculation. This titration gave a figure of $10^{6\cdot0}$ $LD_{50}$/0·03 ml.; thus the monkey received approximately 3 million mouse $LD_{50}$. The monkey was bled daily for 10 days and the serum so obtained titrated in adult mice inoculated by the IC Route. The results given by these titrations are shown below:

| *Hours Post-Inoc.* | *Titre as Mouse $LD_{50}$/1·0 ml. Serum* |
|---|---|
| 24 | 1 mouse of 5 inoculated with undiluted serum died |
| 48 | $10^{3\cdot7}$ |
| 72 | $10^{5\cdot9}$. Monkey had temperature of 103·8°F |
| 90 | $10^{4\cdot5}$ |
| 115 | No activity from this point onwards. |

The only reaction shown by the monkey was a fever of 103·8°F on the afternoon of the 3rd post-inoculation day, at which time it was hyper-excitable. Three weeks after the inoculation MR 1131 was bled and its serum was found to contain neutralizing antibodies to Lunyo virus.

Two grivet monkeys, *Cercopithecus aethiops centralis* Neuman Nos. 1139 and 1140, which had been tested and found non-immune to Lunyo virus, each received 1·0 ml. of 17th-passage mouse-brain suspension that gave a titre of $10^{5\cdot8}$ when inoculated IC into mice. They therefore received a dose of active virus comparable in magnitude to that received by the rhesus monkey, but the virus was derived from stock that had undergone a further 12 mouse passages. These monkeys were bled daily for 14 days and titrations were set up with each specimen obtained. During this period neither monkey showed any sign of a reaction to the inoculum, nor was any virus recovered from their serum. Both monkeys were subsequently shown to have produced neutralizing substances for Lunyo virus.

*Histopathology*

Samples of brain taken from mice infected with early-passage Lunyo material showed an encephalitis of viral type, the only remarkable feature being the presence in the Cornu Ammonis of intranuclear eosinophilic inclusion-bodies, morphologically indistinguishable from those seen in sections of RVF-infected liver. No inclusions could be found in sections of brain prepared from mice inoculated with either viscerotropic or neurotropic RVF.

It has been shown[9] that suspensions containing a high proportion of complete RVF virus produce many blood lakes but few eosinophilic degenerating hepatic cells similar to the Councilman body of yellow fever. As, however, the proportion of incomplete virus in the suspension increases, the blood lakes become fewer and the Councilman-like bodies more numerous.[9] With the viscerotropic strain of Lunyo virus, even when the liver samples are taken from mice with blood titres as high as $10^{8\cdot5}$ $LD_{50}$/0·03 ml., which according to Mims[10] are exceedingly unlikely to contain much incomplete virus, the most prominent feature of the histological picture in the liver is the very great quantity of the degenerate eosinophilic material. This is very much greater in amount than could be produced experimentally by the deliberate inoculation of mice with suspensions containing high proportions of incomplete virus. In those instances in which liver damage occurred after the IC inoculation of Lunyo virus as isolated, the histological picture seen in the liver sections was indistinguishable from that produced by the viscerotropic type.

*The possible isolation of Lunyo virus from a specimen of human blood*

In February 1956 G.O., one of the African mosquito-colony attendants, reported sick with symptoms very suggestive of an infection by RVF. He showed a biphasic fever and virus was isolated from blood taken at both peaks of the fever. The only unusual symptoms he reported were dizziness, disturbances of vision and intense pain in the groin. G.O. has little occasion to enter the laboratory building and, if he should do so, he never enters the wing

containing infective material. It is therefore thought unlikely that his infection could have been accidentally acquired in the laboratory. An important point, however, is that the mice inoculated with G.O.'s blood produced signs of nervous involvement and the histological picture seen in their livers was typical of the Lunyo strain of virus.

We were fortunate in having a control for the above-mentioned case in that, 2 weeks after G.O. became ill, A, one of the sterilizing-room attendants, reported sick with what is almost certainly a laboratory infection of RVF resulting from the handling of glassware which had been employed in an experiment with highly infective RVF serum. The course of A's illness was typical of RVF, as was the behaviour of the mice inoculated with his serum.

Serum from both these men was shown to neutralize not only strains of Lunyo and RVF viruses but also the neuro-adapted RVF which, as mentioned earlier, could not be neutralized in an adult mouse IC test by antisera to either Lunyo or RVF.

*Haemagglutination by Lunyo virus*

Mims and Mason[11] report that RVF serum containing $10^{6\cdot7}$ $LD_{50}/0\cdot5$ ml., in the absence of much incomplete virus, constituted 1 haemagglutinating unit, and also that the infective and haemagglutinating particles are probably identical. In spite of this, Lunyo viscerotropic type of serum containing as much as $10^{9\cdot7}$ $LD_{50}/0\cdot5$ ml. of virus failed to yield a haemagglutinin.

## SUMMARY AND DISCUSSION

The isolation of an agent from locally caught mosquitoes (see Part I) and certain of its properties (see Part II) have been described. At first it was thought that the agent might be a strain of Rift Valley fever (RVF) virus because it produced changes in the liver of infected mice. Attempts to neutralize the agent with potent immune sera to RVF failed, as did further attempts with immune sera to 12 other viruses. The virus produces a characteristic pattern of behaviour in adult mice when inoculated intracerebrally; they first become hyperactive and later exhibit an obsessional eating phase during which any available material is swallowed, causing the stomach to become grossly distended; in many instances the mice attack their own throats or limbs, frequently inflicting severe damage; death is usually accompanied by convulsions.

It was found that the agent could not easily infect adult mice when inoculated by the intraperitoneal route, but on selective intraperitoneal passage of liver or blood a viscerotropic type of virus was soon obtained. This readily infected mice by the intraperitoneal route, and cross-neutralization tests carried out with it against classical RVF virus showed a quantitative reciprocal cross, from which it appears that they are different strains of the same agent. This supposition is supported by the findings of Dr. Max Theiler of New York, who carried out complement-fixation tests with the agent. Further differences, however, were found between the Lunyo and RVF strains; some of these are mentioned below.

The Lunyo and RVF strains produce different histological pictures in mice. An isolation was obtained from a human case and, on the basis of the behaviour of the mice inoculated with blood from this patient, and the histological picture produced in them, it is felt that the virus isolated is probably the Lunyo strain. Previous attempts to obtain a transmission of RVF virus by the bite of the mosquito *A. aegypti*[6,7] have failed, though it would appear that this mosquito readily transmits Lunyo virus. However, passage of viscerotropic-adapted Lunyo virus through a mosquito results in its return to the neurotropic type. A similar tendency for classical RVF to adapt very rapidly to a neurotropic variant was seen when it was passed through a local rodent, *Arvicanthis abyssinicus nubilans* Wroughton. This fact was not reported in the paper concerning these experiments, which dealt entirely with the ability of *A. abyssinicus* to circulate RVF and the possibility that it was a natural host in this area.[12]

In view of these facts it is suggested that Lunyo virus is a variant of classical RVF virus, changes in which may have been brought about by the local conditions, and it is not unlikely that, if this is true, other types of variation might occur elsewhere. As an example of this, retinal haemorrhage in man has been reported as a complication of RVF in the Union of South Africa,[13] whereas it has never been reported from other areas. As another possible example, classical RVF virus is extremely stable[8] whereas Lunyo virus and a strain of RVF isolated in Tongaland, South Africa, are unstable under similar conditions of storage.[14]

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